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硕 士 学 位 论 文

红发夫酵母虾青素的分离纯化
及虾青素免疫机理的研究

Separation and purification of astaxanthin from *Phaffia*
rhodozyma and the immunomechanism of astaxanthin

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摘 要

虾青素是一种具有极强抗氧化和抗衰老活性的类胡萝卜素，被誉为“超级维生素E”，在医药、保健和化妆品等方面有着广泛的应用价值。为了满足日益增加的市场需求，急需进一步降低虾青素的生产成本，目前市售虾青素主要来源是雨生红球藻，而对于红发夫酵母来源虾青素的分离提纯却鲜有报道；另一方面，虽然虾青素强大的生理功能已逐渐被人所知，但对其免疫功能及其作用机理尚未有深入的研究报道，为使虾青素在人类健康方面更好地发挥作用，对其免疫机理的研究也是非常必要的。

本文首先对红发夫酵母虾青素的分离纯化进行研究。采用高压匀浆破碎法破碎红发夫酵母细胞，考察不同破碎压力、破碎次数和处理量对细胞破壁及虾青素萃取率的影响；结果表明，采用 4 mL 乙醇萃取 0.03 g 菌体，并在 40 kpsi 压力下破碎 4 次时，其虾青素萃取率最高可达 71.93%。在此基础上，对虾青素的萃取相转移条件进行优化，结果表明采用 5 mL 乙醇萃取液与 4 mL 正己烷、5 mL 双蒸水混合时，虾青素的相转移率可达 98.27%；最后，进一步对硅胶柱层析分离纯化萃取相中的虾青素进行了研究，结果表明最佳分离条件为使用 20 g 100-200 目硅胶，装柱 20 mL，以 8 mL 虾青素浓度为 $29.29 \text{ mg} \cdot \text{L}^{-1}$ 的正己烷相转移液进样，采用正己烷/丙酮=95/5 (v/v) 的洗脱剂洗脱前三个杂质条带后，再使用正己烷/丙酮=90/10 (v/v) 的洗脱剂洗脱虾青素条带，最终可以得到纯度大于 95.5% 的虾青素。

其次，本文以小鼠和大鼠为研究对象，考察虾青素对生物体免疫性能的影响。通过对小鼠灌胃低 ($4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$)、中 ($20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) 和高剂量 ($100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) 的虾青素，考察虾青素对实验组与对照组的免疫器官脏器指数、巨噬细胞吞噬功能和血清抗体水平。结果表明，各剂量组小鼠血清抗体水平均显著升高 ($P < 0.01$)，中剂量组小鼠脾脏指数显著升高 ($P < 0.05$)，中、高剂量组小鼠的胸腺指数和吞噬系数均显著升高 ($P < 0.05$)。采用腹腔注射 D-半乳糖的方式建立大鼠的致衰老模型，考察虾青素灌胃对其免疫器官脏器指数及其形态的影响。结果显示，衰老灌胃虾青素组与衰老对照组相比胸腺指数显著升高 ($P < 0.01$)，同时 D-半乳糖诱

导致使的肝脏、胸腺和脾脏细胞损伤得到明显改善。由此可以看出，虾青素可提高非特异性免疫和体液免疫功能，该结果为虾青素免疫机理的进一步研究奠定了基础。

最后，为探讨虾青素的免疫机理，本文对动物实验中四组大鼠的血清进行蛋白质组学分析。通过一维电泳考察了不同方法对血清高丰度蛋白的去除效果，结果表明使用Bio-rad血清白蛋白清除试剂盒处理血清样品的效果最佳；此外，通过LC-MS/MS分析，发现无论正常组大鼠还是致衰老模型组大鼠，在灌胃虾青素后，大鼠血清中 α B晶状体蛋白、细胞外基质蛋白-1、Ras相关蛋白Rab-27B和激肽原蛋白-1等与肿瘤等恶性病相关蛋白表达下调，而补体第二激活途径中备解素蛋白P分子和补体C8蛋白分子的表达量上调；因此推测虾青素促进大鼠机体免疫功能的机制为：抑制恶性细胞增殖，激活免疫系统T细胞；且通过强化免疫系统的补体第二激活途径，在感染早期便发挥免疫作用。

关键词：虾青素；纯化；免疫指数；差异蛋白

Abstract

Astaxanthin, one kind of high-effective antioxidative and antiaging carotenoid, is well-known as "super vitamin E" and widely utilized in pharmaceuticals, cosmetics and health care fields. In order to meet the increasing market demand, it is very urgent to reduce the cost of astaxanthin production. *Haematococcus pluvialis* is the main source of commercial astaxanthin currently. However, no special attention has been paid to evaluate the extraction of astaxanthin from *Phaffia rhodozyma*. On the other hand, the immune function of astaxanthin, as well as its related mechanisms has not been reported yet, though the physiological function of astaxanthin has been known gradually. To make astaxanthin an more important role in human health, it's also necessary to reveal the immune mechanism of astaxanthin.

Firstly, the separation and purification of astaxanthin from *Phaffia rhodozyma* were studied. High pressure homogenization was applied to disrupt the cell of *Phaffia rhodozyma*. The effects of pressure, cycle number and biomass dosage on cell disruption and astaxanthin extraction were investigated. It was found that the best astaxanthin extraction rate (71.93%) occurred when the pressure, cycle number and biomass dosage were 40 kpsi, 4 passes and 0.03 g dry biomass in 4 mL ethanol, respectively. To remove most of impurities from extraction, conditions on the phase transfer extraction of astaxanthin were investigated. The results showed that the highest phase transfer rate (98.72%) was obtained when using the mixed system composed of ethanol extract, hexane and water with the ratio of 5:4:5. Moreover, further separated and purified of astaxanthin from hexane extraction by silica gel column chromatography were studied. The results showed that the ultimate purity of astaxanthin could be up to **95.5%**, and the optimal operation conditions were as follows: Filled the separation column with silica gel of 100-200 mesh, loaded with 8 mL hexane extraction of which the astaxanthin concentration was $29.29 \text{ mg} \cdot \text{L}^{-1}$ at every turn, and collected astaxanthin with hexane-acetone (90/10, v/v) after eluting the premier 3 bands with hexane-acetone (95/5, v/v) at last.

To evaluate the effect of astaxanthin on immune function, experiments in mice and rats were conducted. The immune organs indexes, macrophage phagocytic function and serum antibody level of mice were studied through different dose gavage

(low dose: $4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, mid dose: $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, and high dose: $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). Compared with the control group, the antibody level in all treated groups ($P < 0.01$), the spleen indexes in mid-dosage group ($P < 0.05$), the thymus indexes and the phagocytosis indexes in both mid and high-dosage group ($P < 0.05$) were significantly increased. The aging rats were made by injecting D-gal into abdominal cavity continually, and the impact of gavaging astaxanthin on the immune organ indexes and morphology were observed. The results showed that the thymus index of aging model rats was significantly raised ($P < 0.01$) by gavaging astaxanthin, and the cell injury of liver, thymus and spleen induced by D-galactose was obviously improved. This study demonstrated astaxanthin can enhance nonspecific immunity and humoral immune function, which laid a foundation for study on immune mechanism of astaxanthin.

Finally, in order to clarify the immune mechanism of astaxanthin, rats serum of four groups were further analysed by proteomics. The effects of different methods for high-abundance proteins removal from rats' serum were detected by one-dimensional gel electrophoresis. The results showed that the best way to eliminate high-abundance proteins was to use the Bio-rad serum albumin clean-up kit. Moreover, the differentially expressed proteins of both normal rats and aging model rats after lavaging astaxanthin were analysed by LC-MS/MS. It was found that Alpha-crystallin B chain, extracellular matrix protein-1, Ras-related protein Rab-27B and Kininogen-1 protein were down-expressed, while complement factor properdin and complement component 8 were up-expressed. These results indicated that the immune-enhancing mechanism of astaxanthin probably related to inhibiting the proliferation of malignant cell, activating the function of T cells in immune system, and enhancing alternate activating pathway of complements of immune system in the early period of infection.

Key words: Astaxanthin; Purification; Immunity indexes; Differential proteins

第一章 绪论

1.1 虾青素概况

1.1.1 虾青素的结构及理化性质

虾青素 (astaxanthin)^[1], 学名 3,3'-二羟基-4,4'-二酮基- β,β' -胡萝卜素, 是类胡萝卜素的含氧衍生物, 属于酮式类胡萝卜素, 分子式 $C_{40}H_{52}O_4$, 分子量 596.86, 结构由四个异戊二烯单位以共轭双键形式相连接, 共含有 11 个共轭双键, 两边是 2 个含氧的六元环, 羟基处于羰基的 α 位, 构成了 α 羟基酮的结构, 具体如图 1.1 所示:

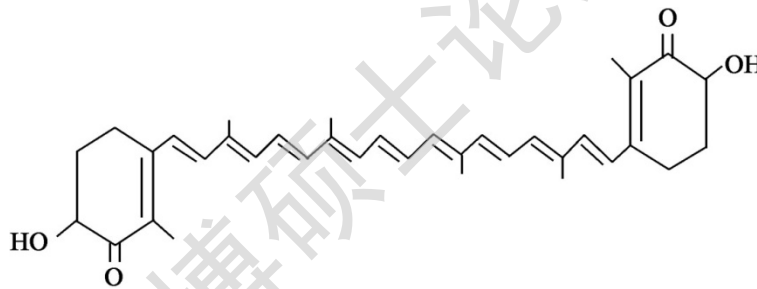


图 1.1 虾青素的结构式

Fig.1.1 Structural formula of astaxanthin

虾青素分子内的共轭双键数达到 13 个, 这样一个长的共轭体系降低了电子能级差, 使得虾青素能吸收可见光区的光而呈现粉红色, 而且虾青素在与蛋白结合后会呈现蓝色等色泽, 晶体状虾青素是一种精细深紫褐色粉末。在三氯甲烷中的最大吸收波长为 $\lambda_{\max}=489\text{ nm}$, 乙醇中 $\lambda_{\max}=478\text{ nm}$, 丙酮中 $\lambda_{\max}=480\text{ nm}$, 二甲基亚砷中 $\lambda_{\max}=492\text{ nm}$, 石油醚中 $\lambda_{\max}=474\text{ nm}$ 。

虾青素熔点大约为 224°C , 但由于分子结构中共轭多烯结构, 所以极其不稳定, 容易受光、热、酸、氧的影响而破坏结构。

由于虾青素分子中仅存在两个羟基, 而疏水基团大量存在, 因此虾青素不溶于水而溶于二氯甲烷、氯仿、丙酮、苯、二硫化碳等有机溶剂, 同时虾青素是一

种弱极性的化合物，它不溶于水，在极性弱的有机溶剂中溶解性较大，如在氯仿中溶解度约为 $10 \text{ g} \cdot \text{L}^{-1}$ ，在极性强的有机溶剂中溶解较小，如在丙酮中为 $0.2 \text{ g} \cdot \text{L}^{-1}$ 、二甲基亚砷中 $0.5 \text{ g} \cdot \text{L}^{-1}$ 。另外，由于分子内含有羟基，虾青素也可以和蛋白质或脂肪酸等化合成酯，根据其酯化程度的不同，可分为虾青素单体、虾青素单脂、虾青素双脂。单体虾青素不稳定，在细胞中多以脂质形式存在。

由于分子中存在很多碳碳双键^[2]，因此虾青素有多种顺反异构体，主要构象为全反式结构、9 位碳和 13 位碳上的顺式异构，如图 1.2 所示。Liu 等人^[3]的研究认为，在抗氧化能力方面，不同异构体的表现不同，其抗氧化能力为：全反式 < 13-顺式 < 9-顺式。

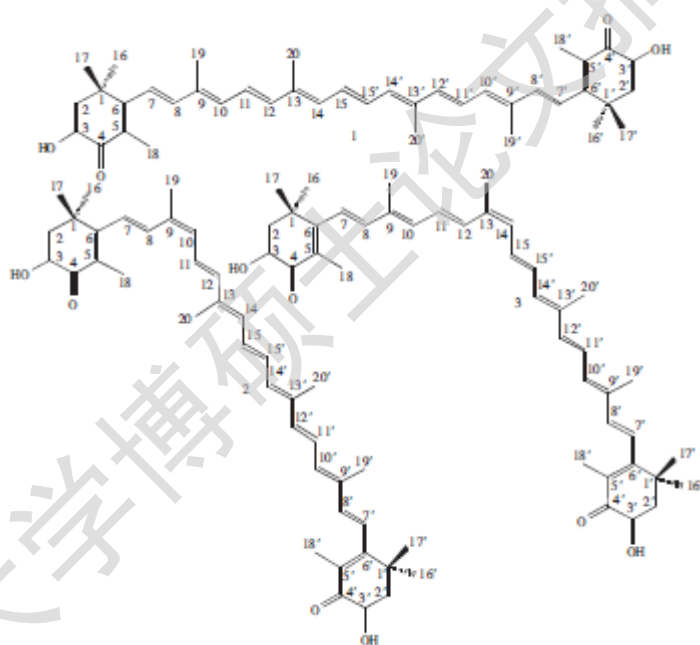


图 1.2 虾青素顺反异构体结构图：全反式、9-顺、13-顺

Fig. 1.2 Structures of cis and trans isomers of Astaxanthin: all-trans, 9-cis, 13-cis

虾青素分子中 3 位 C 和 3' 位 C 是两个手性碳原子，因此虾青素存在 3 种立体异构体，即 3R,3'R、3S,3'S 和 3R,3'S 异构型态，分别称为右旋、左旋、消旋，如图 1.3 所示。不同生物体中发现的虾青素，其旋光异构体构象所占比例不尽相同^[4-5]。

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